Marginal Dietary Copper Restriction Induces Cardiomyopathy in Rats[1,2]

Yan Li,*[3] Lipeng Wang,†[3] Dale A. Schuschke,** Zhanxiang Zhou,* Jack T. Saari,‡ and Y. James Kang*†[4]

*Departments of *Medicine, †Pharmacology and Toxicology, and **Physiology and Biophysics, University of Louisville School of Medicine, Louisville, KY 40202; and ‡U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202*

ABSTRACT Prior studies have provided evidence of marginal dietary copper restriction in humans. The present study was undertaken to examine in a rat model the effect of a long-term marginal dietary Cu deficiency on the heart. Male adult Sprague-Dawley rats were fed AIN-76 diet containing 6.0 (control), 3.0, or 1.5 mg Cu/kg starting at 11 wk of age. Groups of rats were killed at 6, 9, 12, 15, or 18 mo after initiation of feeding, and the same experiment was repeated once. The only systemic change induced by marginal dietary Cu restriction ($P < 0.05$) was depression of organ Cu concentrations in rats fed 1.5 mg Cu/kg diet. Cardiac pathological manifestations in rats fed lower Cu diets were evidenced by histopathological, ultrastructural, and functional alterations. Myocyte hypertrophy and excessive collagen deposition in the heart occurred in rats fed 1.5 mg Cu/kg diet. Ultrastructural changes, including increased number and volume of mitochondria along with disruption of cristae structure, diastolic and systolic dysfunction, and electrocardiograph alterations, occurred in rats fed 1.5 or 3.0 mg Cu/kg diet. These results demonstrate that, in the absence of most indications of systemic Cu deficiency, heart morphology and function are sensitive to marginal Cu deficiency. J. Nutr. 135: 2130–2136, 2005.

KEY WORDS: • *cardiac dysfunction* • *cardiomyopathy* • *copper deficiency* • *myocardial fibrosis* • *myocyte hypertrophy*

Marginal copper restriction in humans, defined here as consumption of a diet resulting in less than the Recommended Dietary Allowance (RDA),[5] has been identified from surveys of food consumption (1–3). The potential relevance of this to the cardiovascular system is illustrated by the findings of a human study on the effects of low-Cu diets (4). Among 24 human subjects consuming a typical American diet low in Cu (1.03 mg/d), some developed cardiac abnormalities (4). For instance, 1 mildly obese subject sustained a myocardial infarction 4 wk after consuming a starch-based diet low in Cu, and 2 subjects experienced severe tachycardia 7 or 10 wk after consuming a fructose-based diet low in Cu. Another subject experienced a type II, second-degree heart block 11 wk after consuming the starch-based diet. Other studies have shown that Cu concentrations in the aorta and coronary arteries decrease with age (5–7), and lower Cu concentrations were found in the heart in elderly people (8). In general, higher concentrations of Cu are associated with better vascular structure (9). Accumulated data support the speculation that experimental animals respond to diets low in Cu similarly to humans (9,10). Thus, it is relevant to determine the effect of long-term marginal Cu deficiency on cardiac morphological and physiological changes in animal models.

Several animal studies have demonstrated cardiovascular defects using diets that simulated the marginally low Cu content of some Western diets. Hunsacker et al. (11) demonstrated that marginal Cu nutriture (2 mg/kg diet) begun in utero and extended to 4 mo of age caused morphologic abnormalities of the aorta in rats that had no other overt signs of Cu deficiency. Schuschke et al. (12) showed that rats fed 3 mg Cu/kg diet from weaning suffered impaired coagulation after 5 wk of feeding, again with no overt signs of Cu deficiency. Wildman et al. (13) performed a study using rats fed diets containing low concentrations of Cu (1.3 and 2.8 mg/kg) from midgestation through 5.5 mo of age. They found that marginal Cu deficiency compromised myocardial ultrastructure, as indicated by altered basal laminae and mitochondria and the appearance of glycogen granules and lipid droplets, all in the absence of cardiac hypertrophy. We aimed to extend and complement the latter important findings in several ways. First, we sought to distinguish effects occurring in adult rats from those occurring during development and therefore initiated feeding of marginally Cu deficient diets similar to those used previously, but at 11 wk of age. Second, to address the effects of aging, we thought it important to extend the period of exposure to the marginal Cu deficient diets and therefore

[1] Supported by USDA Grant 2002–35200-11573, NIH Grant HL63760, and USDA Current Research Information System Project No. 5450-51000-038-00D.

[2] Mention of trade names or commercial products in this article is solely to provide specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

[3] Authors Y.L. and L.W. contributed equally to this study.

[4] To whom correspondence should be addressed. E-mail: yjkang01@louisville.edu.

[5] Abbreviations used: ECG, electrocardiogram, electrocardiograph; H/E, hematoxylin and eosin; LVEDP, left ventricular end diastolic pressure; $-dP/dt$, maximum rate of pressure decline during contraction; $+dP/dt$, maximum rate of pressure development during relaxation; PVC, premature ventricular contraction; RDA, Recommended Dietary Allowance.

Manuscript received 2 February 2005. Initial review completed 22 February 2005. Revision accepted 10 June 2005.

extended the time of feeding to 18 mo, making periodic measurements along the way. And last, we thought it important to examine the functional defects associated with any structural damage that might be evident and therefore included measurements of heart contractility and electrocardiograms (ECGs).

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats, age 10 wk, were obtained from Harlan and maintained at the University of Louisville animal facilities or the USDA Grand Forks Human Nutrition Research Center for 7 d before being assigned to different groups. All of the experimental animals were housed in plastic cages at 22°C on a 12-h light:dark cycle. Rats were divided into 3 groups and fed diets with nominal concentrations of 1.5, 3.0, or 6.0 mg Cu/kg diet. The feeding experiment lasted 18 mo, and 5 rats in each group were killed after 6, 9, 12, 15, and 18 mo of feeding. This feeding experiment was duplicated, with one trial at the University of Louisville (for cardiac functional measurements) and the other at the USDA Human Nutrition Research Center (for cardiac morphology measurements). Cages, feeding jars, and water bottles were rinsed regularly, first with water containing EDTA and then with distilled water. All procedures were approved by the Association for Assessment and Accreditation of Laboratory Animal Care–certified University of Louisville Institutional Animal Care and Use Committee and the Animal Care Committee of the USDA Agricultural Research Service, Grand Forks Human Nutrition Research Center.

The basal diet, an AIN-76 formulation that was modified according to Johnson and Kramer (14), contained casein (200 g/kg), sucrose (386 g/kg), cornstarch (295 g/kg), safflower oil (50 g/kg), and all known essential vitamins and minerals. Six batches of diet for each nominal Cu concentration were made to accommodate the 18-mo duration of each experiment. Dietary Cu concentrations were determined using a dry-ashing procedure (15), followed by dissolution of the residue in aqua regia and measurement by atomic absorption spectrophotometry (Model 503; Perkins Elmer). For rats raised at the University of Louisville, dietary analysis yielded concentration ranges of 1.4–1.9 (nominal 1.5), 2.6–3.8 (nominal 3.0), and 5.1–6.4 (nominal 6.0) mg Cu/kg diet. For rats raised at the Grand Forks Human Nutrition Research Center, ranges were 1.3–1.6 (1.5), 2.3–3.7 (3.0), and 5.3–6.0 (6.0) mg Cu/kg diet.

Tissue Cu and Fe concentrations. In order to monitor overall Cu status in the experimental animals, Cu concentrations in the liver and kidney and Fe concentrations in the liver were determined using inductively coupled argon plasma emission spectroscopy (Model 35608, Thermo ARL-VG Elemental) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide (16).

Electrocardiography. The experimental procedures published previously (17) were followed closely. Briefly, needle electrodes were placed subcutaneously in the extremities to obtain all leads to the electrocardiograph (ECG). The rats were lightly sedated with a single dose of diazepam 20 min before the beginning of the recording to obtain stable ECG recordings in unrestrained rats. ECG signals were band-pass filtered, amplified, digitized (500 Hz/rat), and recorded using an electrophysiograph. During the recording, the chart speed was set at 10 mm/s, 25 mm/s, and 50 mm/s to allow close examination of the changes in waveforms and for arrhythmia. All analyses were performed on full-disclosure 2-h recordings. QRS complex and T wave were visually confirmed. The measurements of PR interval, QRS duration, and QT interval were from lead II. The measurement of R waves was from lead V. All complexes on the screen were ensemble-averaged using the peak of the QRS as a fiducial point. The QT interval of the signal-averaged complex was then determined manually by placing cursors at the beginning of the QRS and the end of the T wave. Because the QT interval was correlated with the RR interval, the QT interval was corrected (QTc) using the formula $QTc = QT/(RR/100)^{1/2}$.

In situ open-chest heart performance analysis. Rats were anesthetized with 10 mL/kg of 0.5% pentobarbital by i.p. injection. A rectal probe was used to measure body temperature. Rats were placed in a supine position and a midline cervical incision was made to expose the trachea and the right carotid artery. After tracheotomy and endotracheal intubation under a dissecting microscope, the right carotid artery was isolated and cannulated with a flame-stretched PE-50 catheter connected to a transducer. The catheter was pushed toward the heart about 1.5 cm and introduced into the left ventricle through the aortic valve. Hemodynamic parameters were then measured by a Digi-Med Heart Performance Analyzer system (Model 210, Micro-Med, Louisville), and data were recorded by a Digi-Med System Integrator (Model 200, Micro-Med,). Measured variables included heart rate, left ventricular systolic pressure, left ventricular end diastolic pressure (LVEDP), left ventricular diastolic pressure, maximum rate of pressure development during contraction ($+dP/dt$), maximum rate of pressure decline during relaxation ($-dP/dt$), time constant, duration of contraction, time to half-relaxation, and duration of relaxation. Data were averaged every 2 s for each measurement.

Assessment of isoproterenol response. Isoproterenol was delivered through a femoral vein catheter with a microliter syringe pump (Harvard Aparatus-22). It was administered at a constant rate of infusion in varying concentrations of 0.08, 0.16, and 0.32 μg/(min · kg body wt) given for a total of 3 min for each dose (0.1 mL/kg body wt). Rats were allowed to recover for 10–15 min before administration of each successive dose. Heart performance under the stimulation of isoproterenol was analyzed by the same surgical procedure described previously (18).

Assessment of morphology changes. Hearts were excised, washed with saline solution, and placed in 10% formalin. Hearts were cut transversely close to the apex in order to visualize the left and right ventricles. Several sections of heart (4–5 μm thick) were prepared and stained with hematoxylin and eosin (H/E) or Sirius red for collagen deposition and visualized by light microscope. For examination by electron microscopy, a tissue sample preparation procedure described previously (19) was used.

Data analysis. Data are expressed as means ± SEM. Body weight, heart weight, heart weight:body weight ratio, organ mineral concentrations, and cardiac function data were analyzed by 2-way ANOVA (dietary Cu vs. months of diet consumption), followed by Tukey-Kramer contrast where appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

Characterization of systemic changes induced by marginal dietary Cu restriction. Variables known to change with severe dietary Cu deficiency [specifically, body weight, heart weight normalized to body weight, hematocrit, and liver Fe concentration (20)] were not affected by the levels of marginal Cu deficiency tested in this study (**Table 1**). The only significant changes induced in rats fed low-Cu diets were decreased Cu concentrations in the liver (Table 1) and kidney (**Tables 1, 2**). Age affected some variables, including body weight, hematocrit and liver Fe concentration. There was no evident interaction between diet and time in any of these variables.

Cardiac histological and ultrastructural changes induced by marginal Cu deficiency. A series of examinations of the myocardial morphology using H/E-stained tissue slides was conducted (**Fig. 1**). Rats fed the diet containing 1.5 mg Cu/kg showed a progressive degeneration in the heart after 9 mo of feeding. Necrotic cardiomyocytes were easily identified, and myocyte hypertrophy was dominant in the myocardium. Interestingly, we observed necrotic cardiomyocytes in the rats fed the diet containing 6.0 mg Cu/kg for 18 mo; however, no hypertrophied cells were identified in these animals. Corresponding to these histopathological changes, there was excessive accumulation of collagens in the myocardium in rats fed the diet containing 1.5 mg Cu/kg for 15 mo, and more extensive myocardial fibrosis in the rats fed 1.5 mg Cu/kg diet for 18 mo (Fig. 1). We also observed excessive accumulation of collagen in the myocardium in rats fed 6.0 mg Cu/kg diet for 18 mo; the extent was the same as that observed in the rats fed

TABLE 1

Effects of dietary Cu and duration of dietary consumption on traditional indices of Cu deficiency in rats examined for cardiac morphology[1,2]

Month	Dietary Cu	Body wt	Heart wt	Hct	Kidney Cu	Liver Cu	Liver Fe
	mg Cu/kg diet	*g*	*g/kg body wt*		*μmol/kg dry wt*		*mmol/kg dry wt*
6	6.0	453 ± 45	2.72 ± 0.11	0.41 ± 0.03	431 ± 49	192 ± 6	6.1 ± 0.3
	3.0	532 ± 39	2.41 ± 0.09	0.44 ± 0.01	438 ± 36	161 ± 5	6.0 ± 0.7
	1.5	533 ± 12	2.48 ± 0.06	0.43 ± 0.01	417 ± 47	167 ± 5	6.4 ± 0.3
9	6.0	518 ± 32	2.58 ± 0.17	0.47 ± 0.02	417 ± 63	154 ± 19	6.7 ± 1.0
	3.0	558 ± 34	2.36 ± 0.10	0.44 ± 0.01	379 ± 47	161 ± 5	6.4 ± 0.4
	1.5	536 ± 32	2.50 ± 0.06	0.43 ± 0.01	324 ± 65	134 ± 13	7.8 ± 0.5
12	6.0	614 ± 36	2.47 ± 0.16	0.45 ± 0.01	426 ± 54	164 ± 2	6.8 ± 0.4
	3.0	611 ± 67	2.35 ± 0.11	0.43 ± 0.01	400 ± 31	168 ± 5	7.9 ± 0.8
	1.5	600 ± 22	2.56 ± 0.06	0.45 ± 0.02	302 ± 31	161 ± 13	8.1 ± 0.5
15	6.0	627 ± 36	2.43 ± 0.06	0.46 ± 0.01	567 ± 148	190 ± 2	7.2 ± 0.8
	3.0	662 ± 48	2.69 ± 0.23	0.42 ± 0.01	475 ± 72	194 ± 9	5.8 ± 1.2
	1.5	658 ± 81	2.48 ± 0.18	0.44 ± 0.01	354 ± 74	162 ± 19	9.1 ± 1.9
18	6.0	643 ± 26	2.75 ± 0.08	0.42 ± 0.02	389 ± 65	200 ± 2	6.4 ± 2.0
	3.0	742 ± 64	2.34 ± 0.17	0.40 ± 0.02	442 ± 39	195 ± 5	6.8 ± 1.6
	1.5	704 ± 51	2.76 ± 0.13	0.38 ± 0.02	260 ± 36	146 ± 11	7.1 ± 1.4
Statistical comparisons[3]				*P*-value			
Diet		NS	NS	NS	<0.02	<0.0002	NS
Month		<0.0001	NS	<0.004	NS	<0.008	NS
Diet × Month	NS	NS	NS	NS	NS	NS	NS

[1] Values are means ± SEM, $n = 5$.
[2] Hct, hematocrit.
[3] NS, not significant, $P > 0.05$.

1.5 mg Cu/kg diet for 15 mo. The analysis of ultrastructural changes using electron microscopy showed slight swelling and disorganization of mitochondria in the myocardium in rats fed 1.5 or 3.0 mg Cu/kg diet for 6 mo and further remarkable changes in the mitochondrial structures in rats fed the same diets for 15 mo. These changes included mitochondrial swelling, disarray, and decreased number or disappearance of cristae. In addition, changes in other organelles were also noted, such as loss of myofibrils, chromatin condensation and margination in the nucleus, and fibrous protein accumulation (**Fig. 2**).

TABLE 2

Effects of dietary Cu and duration of dietary consumption on organ minerals in rats examined for cardiac function[1]

Month	Dietary Cu	Kidney Cu	Liver Cu	Liver Fe
	mg Cu/kg diet	*μmol/kg dry wt*		*mmol/kg dry wt*
6	6.0	427 ± 27	192 ± 13	4.5 ± 1.7
	3.0	356 ± 11	178 ± 3	6.6 ± 2.4
	1.5	327 ± 6	179 ± 17	6.3 ± 2.1
9	6.0	417 ± 50	163 ± 8	6.8 ± 0.7
	3.0	389 ± 39	176 ± 11	6.1 ± 0.5
	1.5	293 ± 13	143 ± 11	6.1 ± 1.4
12	6.0	338 ± 24	167 ± 5	9.0 ± 0.9
	3.0	354 ± 30	178 ± 8	10.9 ± 2.0
	1.5	297 ± 6	179 ± 14	8.8 ± 0.8
15	6.0	331 ± 22	187 ± 8	8.4 ± 0.6
	3.0	359 ± 25	205 ± 9	13.3 ± 0.8
	1.5	307 ± 17	168 ± 14	10.5 ± 0.8
18	6.0	313 ± 17	187 ± 11	10.2 ± 2.7
	3.0	413 ± 49	173 ± 9	7.2 ± 0.5
	1.5	360 ± 27	192 ± 13	11.9 ± 0.8
Statistical comparisons[2]		*P*-value		
Diet		<0.008	NS	NS
Month		NS	<0.05	<0.0001
Diet × Month		NS	NS	NS

[1] Values are means ± SEM, $n = 5$.
[2] NS, not significant, $P > 0.05$.

Marginal Cu deficiency–induced alterations in ECG. ECG analysis (**Table 3**) revealed that rats fed 1.5 mg Cu/kg diet exhibited prolongation of the PR and QT intervals relative to controls ($P < 0.01$). In addition, some, though not all, rats fed 1.5 mg Cu/kg diet showed premature ventricular contractions (PVCs) (**Fig. 3**). One of 5 rats at 12 mo and 2 of 5 rats at 15 mo in this group displayed PVCs. One rat showed PVCs in the group fed 3.0 mg Cu/kg diet for 9 mo. There were no significant changes in ECG in the rats fed 6.0 mg Cu/kg diet.

Myocardial dysfunction induced by marginal Cu deficiency. Assessment of heart functional changes focused on variables indicating both systolic ($+dP/dt$) and diastolic function ($-dP/dt$ and LVEDP). Under unstressed conditions, low-Cu diets did not significantly affect these variables. However, when rats were stimulated with the β-adrenergic agonist isoproterenol, we observed a blunted response of both diastolic and systolic variables at 9 mo in rats fed 1.5 mg Cu/kg diet (**Fig. 4**). In rats fed 3.0 mg Cu/kg diet, diastolic dysfunction was also observed at 9 mo, but systolic dysfunction was not observed until 15 mo (Fig. 4).

DISCUSSION

Reviews of tabulated data from large national surveys (21) as well as data from several peer-reviewed research studies (1–3) provide estimates that adult Cu intakes below the RDA

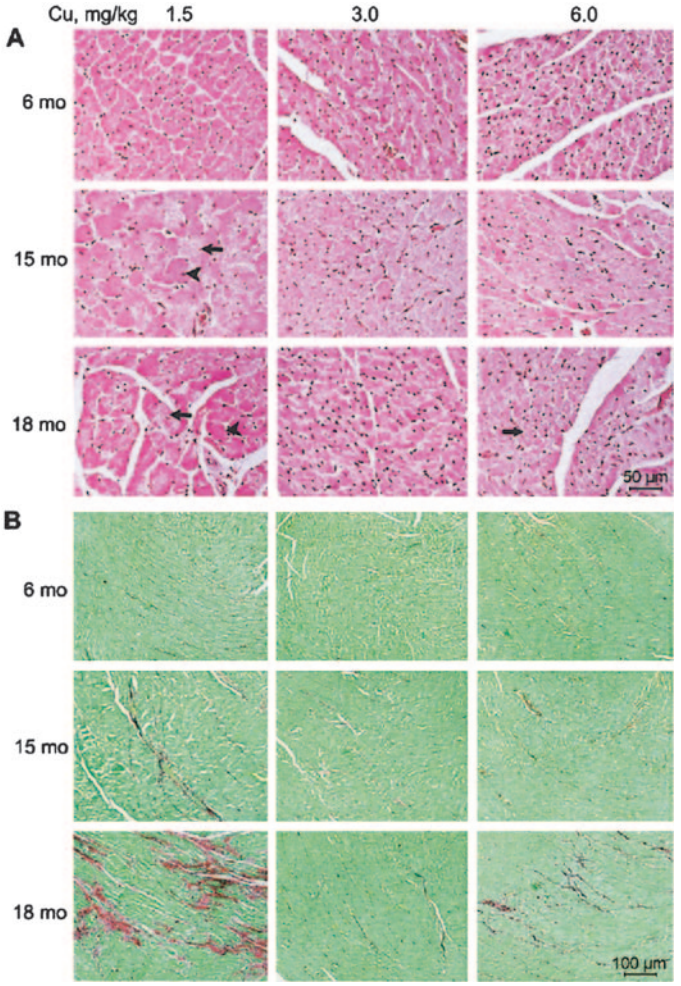

FIGURE 1 Effects of dietary Cu concentration and feeding time on cardiac cellular morphology in rats, examined using light microscopy with H/E staining (*A*) or Sirius red staining (*B*). Arrows indicate necrotic myocytes, arrowheads indicate hypertrophic myocytes, and red staining visualizes collage disposition.

(currently 0.9 mg/d) occur in as much as 60% of the studied populations. With such potentially high numbers of people ingesting less than adequate amounts of Cu, coupled with the known cardiovascular impairment associated with severe Cu deficiency in animals, it is imperative that we understand the effects of marginal Cu deficiency on cardiovascular health. Furthermore, several studies have reported low cardiac Cu concentrations in people who died from ischemic heart disease (22–25). For these reasons, the primary goal of this study was to determine, using rats as a surrogate, whether Cu consumption marginally below the recommended amount results in cardiac structural and functional damage.

Prior animal studies using rats (26–30) or mice (31–33) demonstrated that dietary Cu restriction leads to hypertrophic cardiomyopathy associated with electrophysiological (27–30) and functional abnormalities (33). However, most animal studies were performed under the conditions of acute severe dietary Cu deficiency, raising the question of clinical relevance. An exception is the study of Wildman et al. (13), which examined rats that were fed 2.8 mg Cu/kg diet, defined as marginally Cu deficient compared to the recommendation of 5–6 mg Cu/kg diet. In that study, male offspring were supported by dams fed the marginally Cu deficient diet from

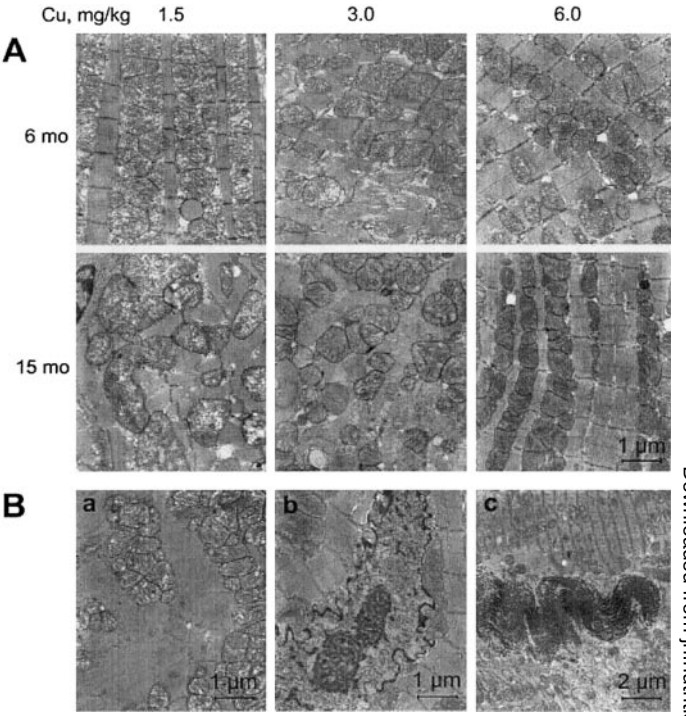

FIGURE 2 Electron micrographs of rat cardiac tissue illustrating the general effects of dietary Cu content and feeding time on myocardial ultrastructure (*A*) and specific effects in rats fed 1.5 mg Cu/kg diet for 15 mo (*B*), including disrupted myofibrils (*a*), nuclear abnormality (*b*), and fibrous protein accumulation (*c*).

midgestation through lactation, then weaned to the same diet until they were 5.5 mo of age. Conventional indices of Cu deficiency did not differ between the rats fed marginally Cu deficient diets and those fed Cu-adequate diets (6.7 mg Cu/kg

TABLE 3

Effects of dietary Cu and duration of dietary consumption on ECG variables in rats[1]

Month	Dietary Cu	PR interval	QRS duration	QT interval
	mg Cu/kg diet		*ms*	
6	6.0	42.8 ± 2.0[b]	17.6 ± 1.0	79.6 ± 4.3[b]
	3.0	44.0 ± 3.2[b]	17.7 ± 1.5	83.3 ± 2.3[b]
	1.5	48.6 ± 5.7[a]	18.0 ± 0.8	90.0 ± 8.1[a]
9	6.0	40.7 ± 0.3[b]	17.9 ± 0.3	80.7 ± 2.6[b]
	3.0	42.6 ± 2.7[b]	18.0 ± 1.1	83.9 ± 3.3[b]
	1.5	50.5 ± 1.8[a]	18.4 ± 0.9	93.8 ± 4.0[a]
12	6.0	40.7 ± 0.4[b]	17.4 ± 0.5	80.6 ± 5.2[b]
	3.0	42.7 ± 3.1[b]	18.5 ± 1.0	82.9 ± 7.6[b]
	1.5	52.5 ± 9.5[a]	18.6 ± 0.6	90.4 ± 6.0[a]
15	6.0	42.0 ± 1.1[b]	17.9 ± 0.6	79.2 ± 1.3[b]
	3.0	41.6 ± 1.5[b]	18.4 ± 0.9	81.8 ± 5.2[b]
	1.5	55.0 ± 6.3[a]	18.3 ± 0.4	90.1 ± 9.6[a]
Statistical comparisons[2]			*P*-value	
Diet		<0.01	NS	<0.01
Month		NS	NS	NS
Diet × Month		NS	NS	NS

[1] Values are means ± SEM, $n = 5$. Means in a column without a common letter differ, $P < 0.01$.
[2] NS, not significant, $P > 0.05$.

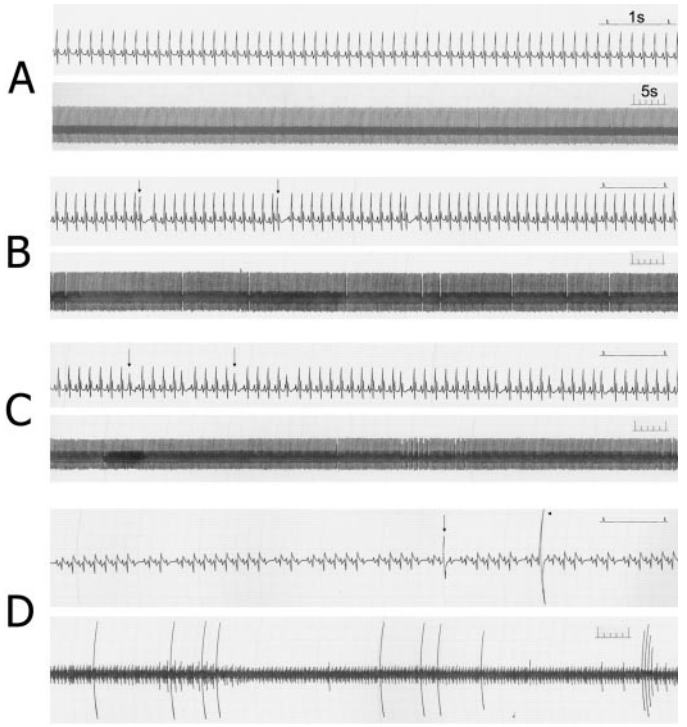

FIGURE 3 Representative ECGs illustrating PVCs in Cu-deficient rats: normal ECG from a rat fed 6.0 mg Cu/kg diet (*A*), compared to ECGs from rats fed 1.5 mg Cu/kg diet for 15 mo (*B*) and 18 mo (*C*, *D*). Examples of PVCs are noted by arrows in each of the 3 abnormal ECGs. The PVCs all show a characteristic absence of P waves. QRS complexes are either larger than normal (*B*, *D*), suggesting a ventricular origin, or smaller than normal (*C*), suggesting a junctional origin. In one instance (*D*), even the regular rhythm is abnormal, showing inverted P and T waves with wide QRS complexes, consistent with a junctional pacemaker.

diet). However, rats fed marginally Cu deficient diets exhibited cardiac structural changes, including increased accumulation of lipid droplets and abnormalities in mitochondria and basal laminae. The observation of cardiac defects at a level of Cu intake likely attainable by humans thus suggested possible clinical relevance of marginal Cu deficiency.

A major difference between the present study and the above study of long-term marginal Cu deficiency (13) was that the prior study initiated the deficiency before birth via dams fed the Cu-deficient diet starting at midgestation. Therefore, the detrimental effects of Cu deficiency on fetal and postnatal development of the heart contributed to the effects of marginal Cu deficiency on the adult heart. The present study eliminated these developmental effects by introducing marginally Cu-deficient diets to adult rats at 11 wk of age. In addition, the observation period was increased (18 mo vs. 5.5 mo).

In acute severe Cu deficiency, the most commonly used index for the cardiac effect is the increased heart weight normalized to body weight. This change was not observed in the present study, even for the longest time (18 mo) of feeding marginally Cu deficient diets. However, histopathological examination did reveal cellular hypertrophy in the myocardium in rats fed 1.5 mg Cu/kg diet for >9 mo. Acute severe Cu deficiency is known to cause myocardial apoptosis (31). Although we did not measure apoptosis in the present study, increasing evidence suggests that apoptosis and hypertrophy are parallel responses to stresses (34–37). For instance, an in vitro study reported that cultured cells underwent apoptosis in response to hydrogen peroxide and at the same time the surviving cells became hypertrophic (36). In vivo studies also showed that myocardial apoptosis occurred simultaneously with the increased volume of the surviving myocytes in response to arsenic toxicity, but without a phenotype of hypertrophy at the organ level (37). Therefore, it appears that the cellular hypertrophy is balanced by cell loss due to apoptosis, so that heart hypertrophy at the organ level is not apparent.

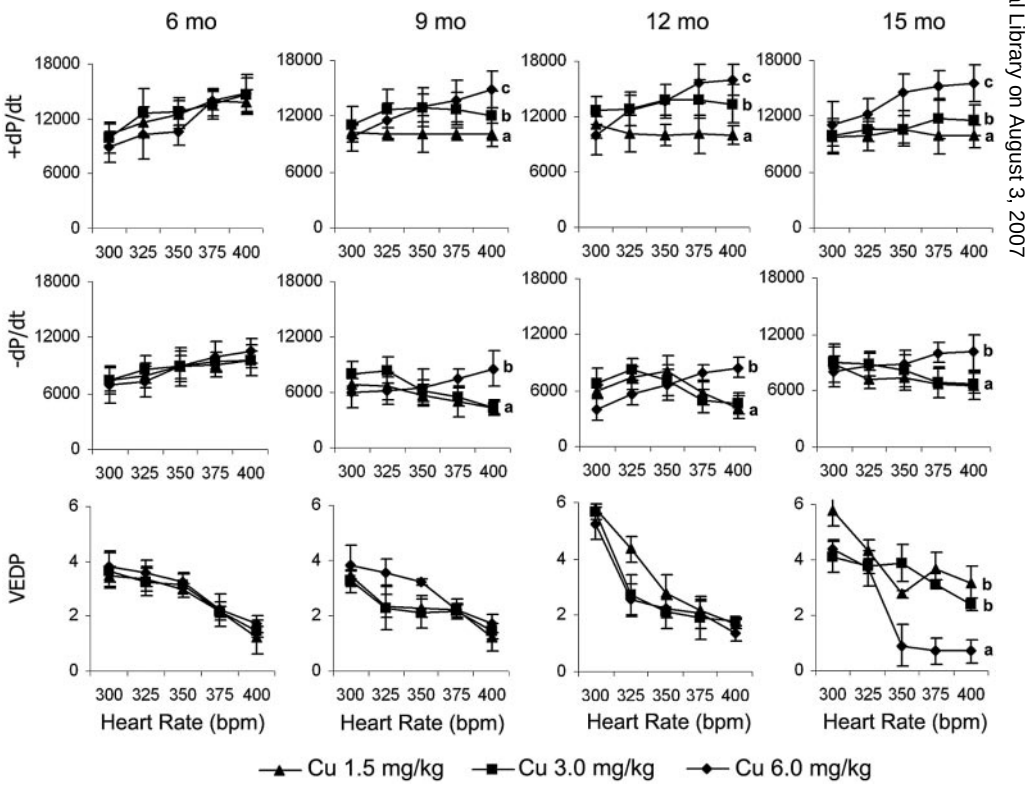

FIGURE 4 Response to adrenergic stimulation of cardiac functional variables in rats fed differing dietary Cu levels for differing times. Isoproterenol was used to stimulate the heart, and changes in $+dP/dt$, $-dP/dt$, or ventricular end diastolic pressure (VEDP) were plotted as a function of the resultant increase in heart rate for each dietary Cu concentration and feeding time. Values in lines (trends of the changes) that do not share the same letter differ significantly, $P < 0.05$.

On the other hand, degenerative progression of the myocardium was evidenced by necrosis and fibrosis at later durations of feeding (Fig. 1). The study of ultrastructural abnormalities by electron microscopy not only confirmed the detrimental effects of long-term feeding of 1.5 mg Cu/kg diet on the heart, but also identified myocardial structural damage in rats fed a diet containing 3.0 mg Cu/kg (Fig. 2A). These and earlier (13) morphological studies emphasize the importance of ultrastructural examination in assessing possible pathology of marginal Cu deficiency.

We also questioned whether the observed morphological alterations were associated with functional abnormalities, which is the ultimate assessment of cardiac injury. The hemodynamic analysis of the left ventricle conducted in the present study provided an examination of changes of cardiac function associated with marginal Cu deficiency under normal and stressed conditions. Heart failure in humans is often diagnosed by the cardiac hemodynamic response to stress, such as that provided by the treadmill test. A change in $+dP/dt$ is indicative of alteration in the systolic function of the left ventricle and is often used to assess the mechanical ability of the heart to generate force for the ejection of blood from the ventricle. With isoproterenol infusion, this variable is increased because of the positive inotropic action of β-adrenergic stimulation. However, the failing heart cannot respond efficiently to such stimulation or may not respond at all, depending on the degree of failure. Analysis of $+dP/dt$ as a function of time demonstrated a blunted response to isoproterenol stimulation after 9 mo of feeding 1.5 mg Cu/kg diet and after 15 mo of feeding 3.0 mg Cu/kg diet, indicating systolic dysfunction. Changes in $-dP/dt$ are indicative of alteration in the diastolic function of the heart. This variable normally increases in response to β-adrenergic stimulation, a response that is blunted in the failing heart. A blunted response to isoproterenol was observed in rats after 9 mo of feeding either 1.5 or 3.0 mg Cu/kg diet. LVEDP is a measurement of ventricular wall compliance and decreases in response to β-adrenergic stimulation. A blunted response of LVEDP to isoproterenol stimulation occurred after 15 mo of feeding either 1.5 or 3.0 mg Cu/kg diet, indicating a stiffening of the myocardial muscle that is consistent with the structural damage described above.

The impaired β-adrenergic response of the Cu-deficient heart triggers the question of a possible link to the known alteration of catecholamine metabolism in the Cu-deficient heart (38–40). However, the depressed catecholamine synthesis with Cu deficiency suggests that a compensatory upregulation of adrenergic receptors should occur, resulting in a greater, rather than reduced, response to isoproterenol. We propose that either the receptor expression is directly compromised by Cu deficiency or, more likely, the downstream contractile mechanisms are impaired, as evidenced by the morphological disruption.

The relative sequence of events indicated that cardiac function was more sensitive than cardiac structure to marginal Cu deficiency and, further, that diastolic dysfunction occurred earlier than systolic dysfunction. These observations demonstrated that, to determine cardiac effects of marginal Cu deficiency, examination of morphological changes, even using electron microscopy, may not be sufficient. Although morphological changes are often observed to precede functional alterations, myocardial dysfunction can apparently occur without evident accompanying morphological changes under certain conditions, such as those presented in this study. This raises the question of whether functional alterations can lead to morphological changes. Both the present study and a previous study of the effects of marginal Cu deficiency on the heart (13) indicated that mitochondria are highly sensitive to Cu deficiency–induced changes. The most common response of mitochondria is swelling and proliferation. Although not observed in the earlier marginal Cu deficiency study (13) it is consistently observed in acute severe deficiency (41,42), and mitochondrial swelling was observed in the longer time frames of the present study. These changes are likely related to high metabolic demand of the myocardium during its adaptation to stress. These pathological changes to the mitochondria would be expected to increase the risk of oxidative damage to the myocardium, thus contributing further to the cardiomyopathy in a positive-feedback manner.

Altered ECGs are commonly found in studies of acute severe Cu deficiency (27–30). The long-term feeding of a marginally Cu deficient diet resulted in prolongation of PR and QT intervals, indicating reduced electrical conduction in both atria and ventricles. A decrease in the heart conduction system in Cu-deficient rats has been reported previously (27,28). Another interesting observation is that some, not all, of the rats fed 1.5 mg Cu/kg diet exhibited PVCs. These ectopic impulses provide further evidence of pathological injury in the heart conduction system. The altered ECGs in marginal deficiency may parallel those found in a human study. In the human study [(4); cited in Introduction] in which subjects consumed a typical American diet low in Cu (1.03 mg/d), 4 of 24 male subjects displayed abnormal ECGs (4). In the human study, pre-existing cardiac conditions cannot be excluded as affecting the outcome. However, the fact that these abnormalities appeared after subjects were fed the controlled low-Cu diet indicates the triggering effect of Cu deficiency. These cardiac effects were reversed after dietary Cu repletion (4). We speculate that the cardiac abnormalities observed in the present study could also be reversed, as is the case for cardiomyopathy in severely Cu deficient mice (18). The above comparisons between animal and human studies suggest that the effects in rats of a diet containing 1.5 mg Cu/kg may well simulate those in humans of a diet providing 1 mg Cu/d. Further studies using the same animal model and the same level of Cu feeding should generate more relevant information regarding the effects caused by diets low in Cu on cardiovascular and other organ systems.

In summary, the present study provided a comprehensive understanding of the detrimental effects of consuming diets low in Cu on the heart through a detailed analysis of the effects of feeding time and Cu level along the model animal's life span. An important general finding is that marginal Cu deficiency can induce myocardial abnormalities in the absence of a conventional measure such as heart enlargement and without the systemic indications of Cu deficiency associated with severe Cu deficiency. Findings also indicate that myocardial alterations induced by marginal Cu deficiency in rats may simulate those found in humans consuming a typical Western diet low in Cu.

ACKNOWLEDGMENTS

The authors thank Sharon Gordon, Gwen Dahlen, Kay Keehr, and Laura Idso for technical assistance.

LITERATURE CITED

1. Klevay, L. M., Buchet, J. P., Bunker, V. W., Clayton, B. E., Gibson, R. S., Medeiros, D. M., Moser-Veillon, P. B., Patterson, K. Y., Taper, L. J. & Wolf, W. R. (1993) Copper in the western diet (Belgium, Canada, U.K. and USA). In: Trace Elements in Man and Animals-TEMA 8 (Anke, M., Meissner, D. & Mills, C. F., eds.), pp. 207–210. Verlag Media Touristik, Gersdorf, Germany.

2. Pang, Y., MacIntosh, D. L. & Ryan, P. B. (2001) A longitudinal investigation of aggregate oral intake of copper. J. Nutr. 131: 2171–2176.

3. Hunt, C. D. & Meacham, S. L. (2001) Aluminum, boron, calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, sodium, and zinc: concentrations in common western foods and estimated daily intakes by infants; toddlers; and male and female adolescents, adults, and seniors in the United States. J. Am. Diet. Assoc. 101: 1058–1060.

4. Reiser, S., Smith, J. C., Jr., Mertz, W., Holbrook, J. T., Scholfield, D. J., Powell, A. S., Canfield, W. K. & Canary, J. J. (1985) Indices of copper status in humans consuming a typical American diet containing either fructose or starch. Am. J. Clin. Nutr. 42: 242–251.

5. Casey, C. E., Guthrie, B. E. & Robinson, M. F. (1982) Copper, manganese, zinc, and cadmium in tissues from New Zealanders. Biol. Trace. Elem. Res. 4: 105–115.

6. Taylor, G. O. & Williams, A. O. (1974) Lipid and trace metal content in coronary arteries of Nigerian Africans. Exp. Mol. Pathol. 21: 371–380.

7. Schroeder, H. A., Nason, A. P., Tipton, I. H. & Balassa, J. J. (1966) Essential trace metals in man: copper. J. Chronic. Dis. 19: 1007–1034.

8. Martin, B. J., Lyon, T. D. & Fell, G. S. (1991) Comparison of inorganic elements from autopsy tissue of young and elderly subjects. J. Trace. Elem. Electrolytes Health. Dis. 5: 203–211.

9. Klevay, L. M. & Medeiros, D. M. (1996) Deliberations and evaluations of the approaches, endpoints and paradigms for dietary recommendations about copper. J. Nutr. 126: 2419S–2426S.

10. Klevay, L. M. (1998) Lack of a recommended dietary allowance for copper may be hazardous to your health. J. Am. Coll. Nutr. 17: 322–326.

11. Hunsaker, H. A., Morita, M. & Allen, K. G. (1984) Marginal copper deficiency in rats. Aortal morphology of elastin and cholesterol values in first-generation adult males. Atherosclerosis 51: 1–19.

12. Schuschke, L. A., Saari, J. T., Miller, F. N. & Schuschke, D. A. (1995) Hemostatic mechanisms in marginally copper-deficient rats. J. Lab. Clin. Med. 125: 748–753.

13. Wildman, R. E., Hopkins, R., Failla, M. L. & Medeiros, D. M. (1995) Marginal copper-restricted diets produce altered cardiac ultrastructure in the rat. Proc. Soc. Exp. Biol. Med. 210: 43–49.

14. Johnson, W. T. & Kramer, T. R. (1987) Effect of copper deficiency on erythrocyte membrane proteins of rats. J. Nutr. 117: 1085–1090.

15. Gorsuch, T. T. (1970) The destruction of organic matter, pp. 28–39. Pregamon Press, Elmsford, NY.

16. Chen, Y., Saari, J. T. & Kang, Y. J. (1994) Weak antioxidant defenses make the heart a target for damage in copper-deficient rats. Free Radic. Biol. Med. 17: 529–536.

17. Kang, Y. J., Li, Y., Zhou, Z., Roberts, A. M., Cai, L., Myers, S. R., Wang, L. & Schuchke, D. A. (2002) Elevation of serum endothelins and cardiotoxicity induced by particulate matter (PM2.5) in rats with acute myocardial infarction. Cardiovasc. Toxicol. 2: 253–261.

18. Elsherif, L., Wang, L., Saari, J. T. & Kang, Y. J. (2004) Regression of dietary copper restriction-induced cardiomyopathy by copper repletion in mice. J. Nutr. 134: 855–860.

19. Kang, Y. J., Chen, Y., Yu, A., Voss-McCowan, M. & Epstein, P. N. (1997) Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J. Clin. Invest. 100: 1501–1506.

20. Klevay, L. M. & Saari, J. T. (1993) Comparative responses of rats to different copper intakes and modes of supplementation. Proc. Soc. Exp. Biol. Med. 203: 214–220.

21. National Academy of Sciences (2001) Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academy Press, Washington, D.C.

22. Anderson, T. W., Neri, L. C., Schreiber, G. B., Talbot, F. D. & Zdrojewski, A. (1975) Letter: Ischemic heart disease, water hardness and myocardial magnesium. Can. Med. Assoc. J. 113: 199–203.

23. Chipperfield, B. & Chipperfield, J. R. (1978) Differences in metal content of the heart muscle in death from ischemic heart disease. Am. Heart J. 95: 732–737.

24. Wester, P. O. (1965) Trace elements in human myocardial infarction determined by neutron activation analysis. Acta Med. Scand. 178: 765–788.

25. Zama, N. & Towns, R.L.R. (1986) Cardiac copper, magnesium, and zinc in recent and old myocardial infarction. Biol. Trace. Elem. Res. 10: 201–208.

26. Allen, K. G. & Klevay, L. M. (1978) Cholesterolemia and cardiovascular abnormalities in rats caused by copper deficiency. Atherosclerosis 29: 81–93.

27. Kopp, S. J., Klevay, L. M. & Feliksik, J. M. (1983) Physiological and metabolic characterization of a cardiomyopathy induced by chronic copper deficiency. Am. J. Physiol. 245: H855–H866.

28. Mao, S., Medeiros, D. M. & Hamlin, R. L. (1999) Marginal copper and high fat diet produce alterations in electrocardiograms and cardiac ultrastructure in male rats. Nutrition 15: 890–898.

29. Klevay, L. M. & Viestenz, K. E. (1981) Abnormal electrocardiograms in rats deficient in copper. Am. J. Physiol. 240: H185–H189.

30. Davidson, J., Medeiros, D. M. & Hamlin, R. L. (1992) Cardiac ultrastructural and electrophysiological abnormalities in postweanling copper-restricted and copper-repleted rats in the absence of hypertrophy. J. Nutr. 122: 1566–1575.

31. Kang, Y. J., Zhou, Z. X., Wu, H., Wang, G. W., Saari, J. T. & Klein, J. B. (2000) Metallothionein inhibits myocardial apoptosis in copper-deficient mice: role of atrial natriuretic peptide. Lab. Invest. 80: 745–757.

32. Kang, Y. J., Wu, H. & Saari, J. T. (2000) Alterations in hypertrophic gene expression by dietary copper restriction in mouse heart. Proc. Soc. Exp. Biol. Med. 223: 282–287.

33. Elsherif, L., Ortines, R. V., Saari, J. T. & Kang, Y. J. (2003) Congestive heart failure in copper-deficient mice. Exp. Biol. Med. (Maywood) 228: 811–817.

34. van Empel, V.P.M. & De Windt, L. J. (2004) Myocyte hypertrophy and apoptosis: a balance act. Cardiovas. Res. 63: 487–499.

35. Bueno, O. F. & Molkentin, J. D. (2002) Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. Circ. Res. 91: 776–781.

36. Chen, Q., Tu, V., Wu, Y. & Bahl, J. (2000) Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes. Arch. Biochem. Biophys. 373: 242–248.

37. Li, Y., Sun, X., Wang, L., Zhou, Z. & Kang, Y. J. (2002) Myocardial toxicity of arsenic trioxide in a mouse model. Cardiovas. Toxicol. 2: 63–73.

38. Gross, A. M. & Prohaska, J. R. (1990) Copper-deficient mice have higher cardiac norepinephrine turnover. J. Nutr. 120: 88–96.

39. Prohaska, J. R., Bailey, W. R., Gross, A. M. & Korte, J. J. (1990) Effect of dietary copper deficiency on the distribution of dopamine and norepinephrine in mice and rats. J. Nutr. Biochem. 1: 149–154.

40. Seidel, K. E., Failla, M. L. & Rosebrough, R. W. (1991) Cardiac catecholamine metabolism in copper-deficient rats. J. Nutr. 121: 474–483.

41. Medeiros, D. M., Davidson, J. & Jenkins, J. E. (1993) A unified perspective on copper deficiency and cardiomyopathy. Proc. Soc. Exp. Biol. Med. 203: 262–273.

42. Medeiros, D. M. & Wildman, R.E.C. (1997) Newer findings on a unified perspective of copper restriction and cardiomyopathy. Proc. Soc. Exp. Biol. Med. 215: 299–313.

JN THE JOURNAL OF NUTRITION